Clin. exp. Immunol. **73**, 484–488

Effect of time, temperature and anticoagulants on *in vitro* complement activation: Consequences for collection and preservation of samples to be examined for complement activation

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(Accepted for publication 4 May 1988)

SUMMARY

The effects of time, temperature, ethylene-diamine-tetra-acetic acid (EDTA), citrate and heparin on *in vitro* complement activation were examined in enzyme immuno assays (EIA) for detection of C3 activation products and the terminal complement complex (TCC). *In vitro* complement activation occurred during coagulation since baseline concentrations of activation products were considerably higher in serum than in plasma. EDTA was more efficient than citrate and heparin in inhibiting *in vitro* activation. Minimal activation was observed in all preparations when samples were kept at 4°C for up to ten days, whereas a very rapid increase in activation products occurred even in EDTA plasma when the temperature was elevated. Based on the data obtained, guidelines for the collection and preservation of samples to be examined for complement activation are given.

Keywords complement complement activation EDTA citrate heparin

INTRODUCTION

Quantification of native complement components and total haemolytic complement activity is usually performed with serum samples. In contrast, measurement of complement activation products generated *in vivo* requires plasma samples since a rapid *in vitro* activation may take place in serum (Mollnes, 1985). Ethylene-diamine-tetra-acetic acid (EDTA) is efficient in binding calcium and magnesium and thereby blocking both the classical and alternative pathway of complement. Thus, EDTA-plasma usually has been recommended for complement activation assays. However, both time and temperature are known to influence the degree of *in vitro* activation, even when the samples are collected directly into tubes containing EDTA.

Sensitive enzyme immuno assays (EIA) for the quantification of complement activation products based on monoclonal antibodies against neoepitopes have been developed in our laboratory (Mollnes *et al.*, 1985; Mollnes & Lachmann 1987; Garred, Mollnes & Lea 1988). The aim of the present study was to examine systematically to what extent time, temperature, heparin, citrate and EDTA influence the degree of *in vitro* complement activation measured in these assays. The results have consequences for the collection and preservation of samples to be examined for complement activation.

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MATERIALS AND METHODS

Collection of Samples

Pools of serum, heparin-, citrate-, and EDTA-plasma were made by collecting blood samples from six healthy donors into dry vacutainer tubes (serum) or standard tubes containing heparin, citrate or EDTA. Whole blood was allowed to clot before serum was pooled and stored whereas the other samples were centrifuged (1,500 ***g***, 15 min) immediately and plasma separated. Samples of each pool were frozen down to −70°C immediately. They were used as baseline samples and to examine the influence of repetitive freezing and thawing. The pools of serum, heparin-, citrate-, and EDTA-plasma were incubated at 4°C and 37°C for 10 days. Samples were frozen down to −70°C at day 1, 2, 3, 6 and 10 and were all examined in one batch.

Complement Activation Assays

C3 activation was measured using a double antibody ELISA described in detail elsewhere (Garred, Mollnes and Lea 1988). Briefly, a mouse monoclonal antibody (bH6) recognizing a C3 neoepitope exposed in C3b, iC3b and C3c was used as capture antibody (coated on the plastic). Test samples and standard (zymosan activated serum) were added and a polyclonal rabbit anti-C3 antiserum was used in the next step. Finally, peroxidase-labelled anti-rabbit Ig was added. The degree of C3 activation (e.g. the amount of this particular neoepitope) is indicated with arbitrary units (AU/ml) referring to the activated standard serum defined to contain 1000 AU/ml. *In vitro* C3 conversion in

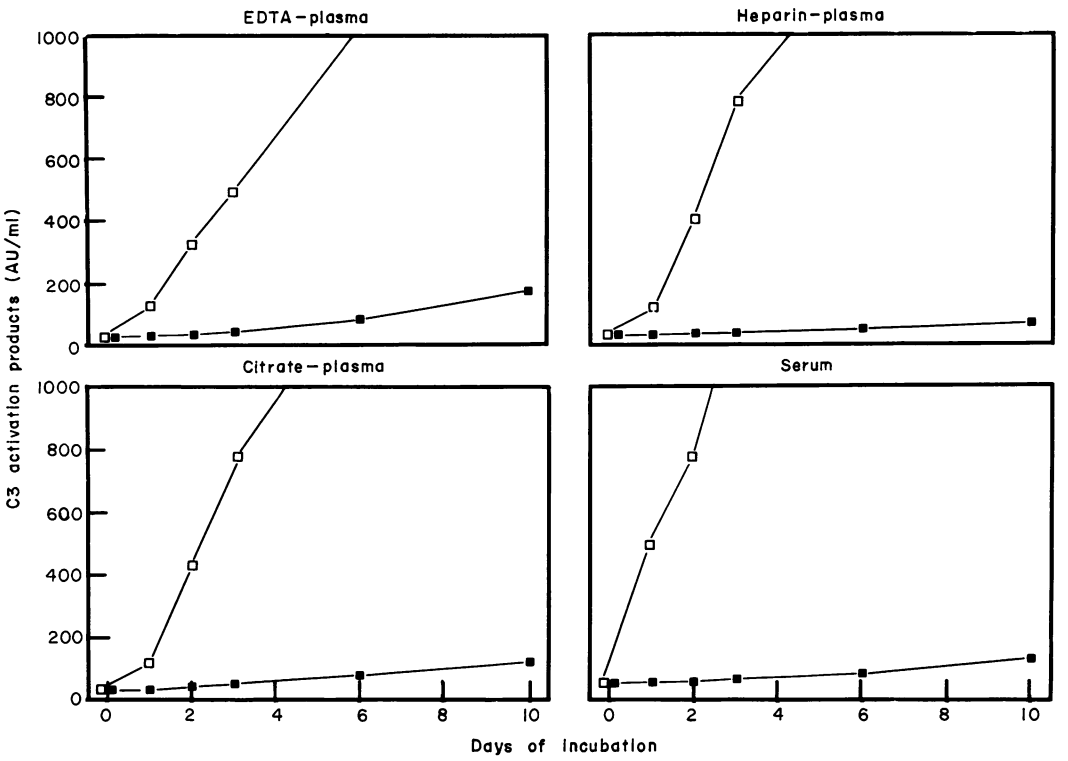

Fig. 1. Generation of C3 activation products (neoepitopes recognized by the monoclonal antibody bH6) for ten days at 4°C (■) and 37°C (□) in pools ($n=6$) of EDTA plasma, citrate plasma, heparin plasma and serum. 1000 AU/ml corresponds to the amount of C3 activation products in the zymosan activated standard.

serum was also examined in a similar C3 activation assay using a rat monoclonal antibody (Clone 9) against a C3g neoepitope (Mollnes & Lachmann 1987). This antibody was a gift from Professor P. J. Lachmann.

Terminal pathway activation was evaluated by measuring the amount of the terminal complement complex (TCC), which is in the form of SC5b-9 in the fluid phase. TCC was quantified using a very similar technique to that described above and has been described in detail previously (Mollnes *et al.*, 1985). Briefly, a monoclonal antibody (aE11) recognizing a C9 neoepitope in TCC was used as capture antibody and a rabbit anti-C5 antiserum as the detection antibody.

All results are given as median of triplicate determinations of each sample. In order to construct the experimental design, two pilot studies were made prior to the present one. The results of the pilot studies were virtually identical to those presented here.

RESULTS

Activation of C3

Baseline values for EDTA-, citrate- and heparin-plasma were similar: 20, 22 and 19 AU/ml, respectively (Fig. 1). In contrast, baseline value for serum was 43 AU/ml. The increase with time at 4°C was slow compared with 37°C, at which a rapid conversion of C3 took place. In serum, the amount of C3 activation products generated at 37°C were comparable to the zymosan activated standard (1000 AU/ml) after incubation for 2 days. This degree of activation was reached after 4 days in citrate- and heparin-plasma. EDTA was most efficient in inhibiting C3 activation at 37°C, 1000 AU/ml was reached after 6 days.

The upper reference limit for a healthy blood donor population ($n=40$) was 22 AU/ml in this assay (Garred, Mollnes & Lea 1988). In EDTA-, citrate- and heparin-plasma this level was reached between day 1 and day 2 at 4°C, and after a few hours at 37°C. In serum, even the baseline value (43 AU/ml) was far above this upper reference limit for C3 activation products in plasma.

C3 neoepitope kinetics

The pattern of activity observed in serum incubated at 37°C differed significantly when the two different monoclonal anti-C3 neoepitope antibodies were compared (Fig. 2). The bH6 antibody, reacting with a neoepitope in C3b, iC3b and C3c, detected an increase in activity during the first 6 days. A small decrease was then observed but thereafter a steady state of high activity was maintained (Fig. 2, left). In contrast, the Clone 9 antibody, reacting with a C3g neoepitope, detected an increase in activity for the first 4 days. Thereafter there was a considerable fall to day 14, at which time a relatively low level of activity was found though it was still higher than the baseline (Fig. 2, right).

Activation of the Terminal Pathway

Generally, the degree of terminal pathway activation *in vitro* was much less pronounced than for C3 (Fig. 3). This is reflected in the five-fold difference in the ordinate scale in Fig. 3 compared to Fig. 1. At 4°C no significant increase was seen in TCC concentration after incubation for 10 days either in serum or in the three plasma samples. However, the baseline value for serum was twice as high as that for plasma. At 37°C a rapid increase in

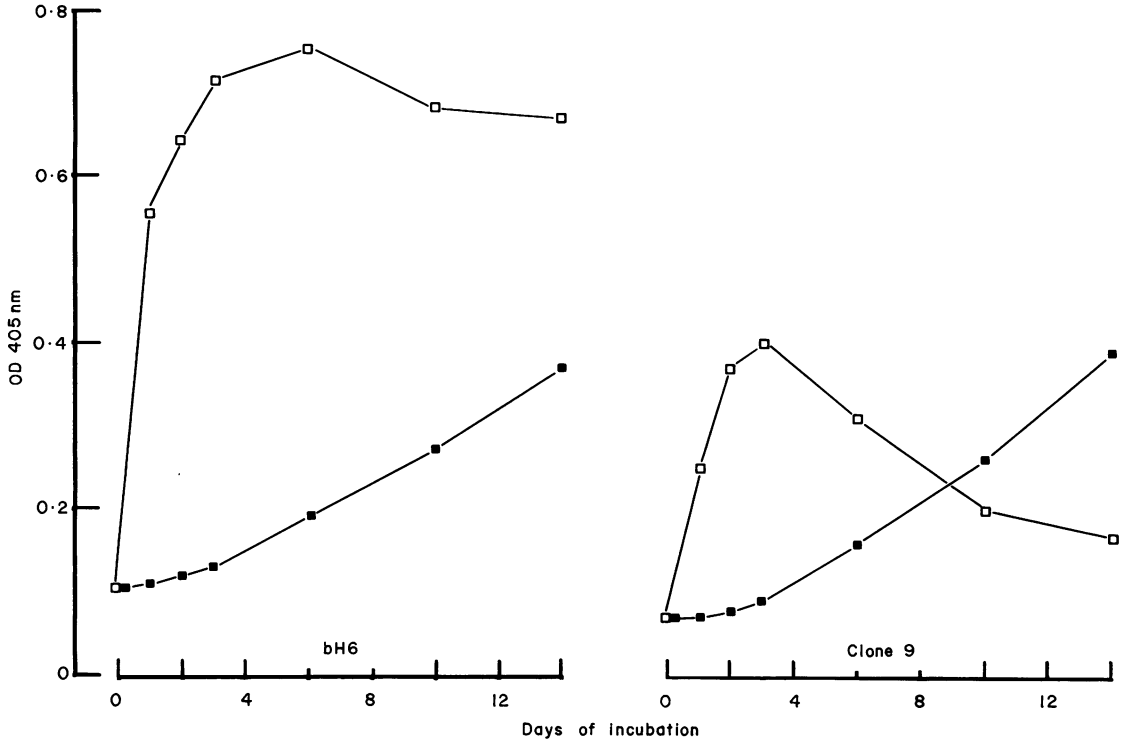

Fig. 2. Generation of two different C3 neoepitopes in serum incubated for 14 days at 4°C and 37°C. MoAb bH6 (left panel) recognizes a neoepitope expressed on C3b, iC3b and C3c whereas Clone 9 (right panel) reacts mainly with a neoepitope on iC3b, less expressed on C3dg and C3g.

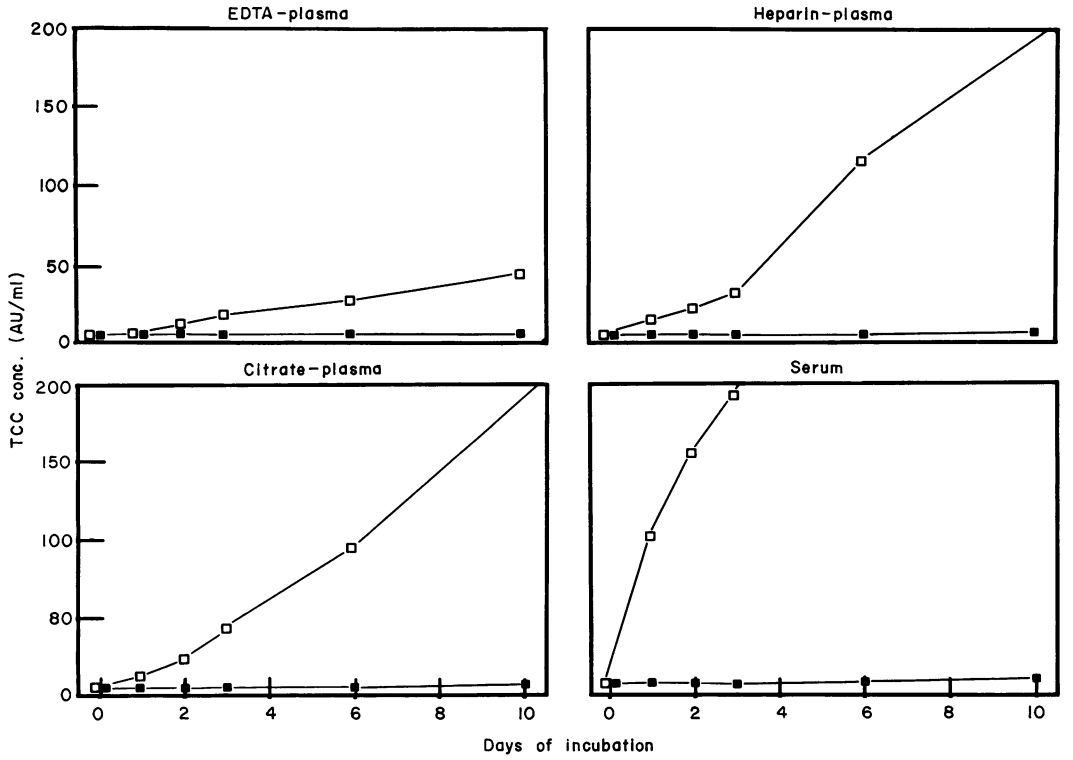

Fig. 3. Generation of TCC under identical conditions as described in Fig. 1. 200 AU/ml (top of the ordinate scale) corresponds to 20% of the amount of TCC present in the zymosan activated standard.

TCC concentration was observed in serum. EDTA most efficiently inhibited activation of the terminal pathway, although a ten-fold increase in TCC concentration was observed from baseline to 10 days (4–40 AU/ml) and the upper reference limit (7·5 AU/ml) was reached during the second day. Citrate and heparin were comparable with respect to inhibition of terminal pathway activation, a 3–4 fold increase in TCC concentration was observed during the first 24 h.

Storage, freezing and thawing

A normal human EDTA plasma pool obtained from 40 healthy blood donors was stored for three years at −70°C, and compared with a similar fresh pool. When examined together these two pools showed exactly the same amount of C3 activation products (15 AU/ml) and TCC (5 AU/ml). Furthermore, freezing and thawing up to four times did not influence the level of C3 activation products and TCC in a pool of normal human EDTA plasma.

DISCUSSION

In vitro complement activation occurs both in the presence and in the absence of activating agents. The latter is due to a spontaneous activation of components as shown for C1 (Ziccardi 1982) and C3 (Pangburn, Schreiber and Müller-Eberhard 1981). Time and temperature influence the degree of *in vitro* activation. Our results strongly emphasize the importance of keeping the temperature low to avoid spontaneous activation.

The effect of citrate and EDTA in preventing coagulation and complement activation is mediated via their calcium and magnesium binding properties. Although heparin selectively interacts with the coagulation system, it also has inhibitory effects on the classical and alternative pathways of complement (Ecker and Gross 1929; Rent *et al.*, 1976; Weiler *et al.*, 1978; Kazatchkine *et al.*, 1981). This explains why the baseline values for activation products were comparable in EDTA-, citrate- and heparin plasma. In contrast the level in serum was considerably higher, consistent with spontaneous activation of complement during coagulation. Increasing the temperature *in vitro* overcame the inhibitory effects present in plasma at 4°C. However, EDTA was more efficient than citrate and heparin, particularly in inhibiting terminal pathway activation.

In all the samples examined there was more pronounced activation of C3 than of the terminal pathway, consistent with the relative inefficiency of fluid phase C5 convertase. Similar observations after *in vitro* activation have recently been observed by others (Bhakdi *et al.*, 1987). Thus, false high values due to *in vitro* activation will more readily occur for C3 activation products than for TCC. On the other hand, low grade *in vivo* activation in the fluid phase can be reflected in increased C3 activation products and in normal amounts of TCC. The degree of dissociation between C3 and the terminal pathway can be further modified by different factors influencing the function of the convertases as discussed for nephritic factors (Mollnes *et al.*, 1986).

Two different patterns appeared when serum was incubated at 37°C and examined with the two monoclonal antibodies bH6 and Clone 9. Whilst bH6 reacts with a C3 neoepitope expressed in C3b, iC3b and C3c (Garred *et al.*, 1988), clone 9 reacts with a neoepitope expressed in iC3b, C3dg and C3g (Lachmann, Pangburn & Oldroyd 1982). In the ELISA method used in the present study, Clone 9 has been shown to react strongly with iC3b compared with C3dg (Mollnes & Lachmann 1987). Our data are therefore consistent with an initial increase in iC3b and a subsequent fall due to further breakdown to C3c and C3dg, although Clone 9 reacted sufficiently with C3dg fragments fixed in immune complexes (Samuel *et al.*, 1986). In contrast, bH6 reacts with comparable affinity with iC3b and C3c (Garred *et al.*, 1988) and the activity therefore remained during incubation. The two assays correlated closely when samples from patients were examined suggesting that iC3b may be the main contributor of free C3 neoepitopes in plasma during *in vivo* activation (Mollnes & Lachmann 1987; Garred, Mollnes & Lea 1988). Both assays are therefore reliable for detection of *in vivo* C3 activation, although Clone 9 will require a more careful preparation of the standard for reproducible results.

In conclusion, we have shown that EDTA inhibits C3 and terminal complement activation in plasma more efficiently than citrate and heparin. Additionally, in order to avoid *in vitro* activation it is most important to keep the sample at a constantly low temperature. Guidelines for preparation and storage of samples to be examined for *in vivo* complement activation should be more strict than the limits defined in this experimental study for two reasons. Firstly, conditions in clinical practice are less easily controlled than those used in experiments *in vitro* and secondly, samples with concentrations of activation products in the upper reference range at the time of venepuncture will exceed the upper reference limit earlier than others. Thus, we recommend the following guidelines: the blood sample should be collected directly into tubes containing EDTA and cooled to 4°C immediately, the plasma should be separated and stored at 4°C until frozen at −70°C, which should occur within 8 h. Once stored safely at −70°C, no significant activation will occur during the first three years.

ACKNOWLEDGMENTS

We are greatly indebted to Ms. Kari Bertelsen for her excellent secretarial assistance. Financial support was given by the Norwegian Women's Health Organization, Nordisk Insulinfond and the Norwegian Research Council for Sciences and the Humanities.

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